Fc fusion Proteins of Human Granulocyte Colony-Stimulating Factor With Increased Biological Activities

Inventors: Lee-Hwei K. Sun, Bill N. C. Sun, and Cecily R. Y. Sun

Background

[0001] Granulocyte colony-stimulating factor (G-CSF) is a 20 kilodalton (kDa) glycoprotein that promotes the proliferation of progenitor cells and induces their differentiation into neutrophils. In addition, G-CSF prolongs the survival of mature neutrophils and activates their functions Human G-CSF (hG-CSF) is produced by monocytes, macrophages, fibroblasts and endothelial cells (see, for example, Moore, *Annu. Rev. Immunol.*, 9:159-191, 1991; Nicola, *Annu. Rev. Biochem.*, 58:45-77, 1991). The biological effects of G-CSF are mediated through its interaction with the G-CSF receptor (G-CSF-Rc) expressed on the surface of bone marrow hematopoietic progenitors and cells of the myeloid lineage. Upon binding G-CSF, the receptor is activated and undergoes homodimerization, followed by phosphorylation of Janus family of tyrosine kinases. Subsequently, a series of intracellular signal transduction events take place, leading to the increase of the number of progenitor cells, their maturation into neutrophils, and further activation of effector functions in mature neutrophils (see, for example, Demetri et al., *Blood*, 78:2791-2808, 1991). Therefore, G-CSF plays an essential role not only in the regulation and maintenance of hematopoiesis, but also in host defense against infection and inflammation.

[0002] Recombinant human G-CSF (rhG-CSF) is widely used in the treatment of patients with neutropenia as a result of receiving chemotherapy. Administration of rhG-CSF is effective in restoring functioning neutrophils to these patients, leading to a decrease of infection-related events. Use of rhG-CSF allows intensified dosing or scheduling of chemotherapeutic agents that may be of benefit to cancer patients. Besides chemotherapy-induced neutropenia, rhG-CSF has been used for the treatment of myelosuppression after bone marrow transplantation, acute leukemia, aplastic anemia, myelodysplastic syndrome, severe chronic neutropenias, and

mobilization of peripheral blood progenitor cells for transplantation (see, for example, Welte et al., *Blood,* 88:1907-1929, 1996).

[0003] The elimination half-life of the serum concentration of rhG-CSF is approximately 3 to 4 h for intravenous or subcutaneous administration. The safety profile and patient tolerance of rhG-CSF are good with medullary bone pain being the only frequent and significant side effect. The relatively low toxicity of rhG-CSF has made it feasible to develop longer-acting derivatives to decrease the inconvenience of the daily or twice-daily injection schedule. Attachment of polyethylene glycol (PEG) to various proteins, including G-CSF, has been reported to yield derivatives with higher *in vivo* potency due to their longer half-lives (see, for example, Zalipsky et al., in *"PEG chemistry: biotechnical and biomedical applications"*, pp. 347-370, 1992). PEG-conjugated proteins usually have considerably lower *in vitro* biological activity than their unmodified parent proteins (Eliason et al., *Stem Cells*, 18:40-45, 2000). The increased *in vivo* potency of these modified proteins is, at least in part, due to decreased removal by the kidney in a manner proportional to their molecular weight (Yamaoda et al., *J. Pharmaceut. Sci.,* 83:601-606, 1994). We unexpectedly discover that it is possible to increase the potency of hG-CSF through prolonging its half-life as well as enhancing its biological activity is to attach the Fc region derived from human IgG at the C-terminus of hG-CSF, as described in this invention.

[0004] Immunoglobulins of IgG class are among the most abundant proteins in human blood. Their circulation half-lives can reach as long as 21 days. Fusion proteins have been reported to combine the Fc regions of IgG with the domains of another protein, such as various cytokines and soluble receptors (see, for example, Capon et al., *Nature*, 337:525-531, 1989; Chamow et al., *Trends Biotechnol.*, 14:52-60, 1996); US patents 5,116,964 and 5,541,087). The prototype fusion protein is a homodimeric protein linked through cysteine residues in the hinge region of IgG Fc, resulting in a molecule similar to an IgG molecule without the CH1 domains and light chains. Due to the structural homology, Fc fusion proteins exhibit *in vivo* pharmacokinetic profile comparable to that of human IgG with a similar isotype. This approach has been applied to several therapeutically important cytokines, such as IL-2 and IFN-α_{2a}, and soluble

receptors, such as TNF-Rc and IL-5-Rc (see, for example, US patents 5,349,053 and 6,224,867). It is desirable to extend the circulating half-life of G-CSF and/or to increase its biological activity by making fusion proteins containing G-CSF linked to the Fc portion of the human IgG protein as disclosed and/or described in this invention.

[0005] Erythropoietin (EPO) derivatives, such as dimers, have been reported. Relative to the EPO monomer, a fusion protein consisting of two complete EPO domains separated by a 3- to 7-amino acid peptide linker exhibited reduced activity (Qiu et al., *J. Biol. Chem.*, 273:11173-11176, 1998). However, when the peptide linker between the two EPO domains was 17 amino acids in length, the dimeric EPO molecule exhibited considerably enhanced *in vitro* and *in vivo* activities (see, for example, Sytkowski et al., *J. Biol. Chem.*, 274:24773-24778, 1999; US patent 6,187,564). The length of the peptide linker between the two hematopoietic growth factors is important, while not bound by this theory, presumably due to its effect on the flexibility of such molecular forms. We find that this approach is generally applicable to other therapeutic proteins, including G-CSF. We'll also refer this to this as a flexible peptide linker.

[0006] In most of the reported Fc fusion protein molecules, a hinge region serves as a spacer between the Fc region and the cytokine or soluble receptor at the amino-terminus, allowing these two parts of the molecule to function separately (see, for example, Ashkenazi et al., *Current Opinion in Immunology*, 9:195-200, 1997). A human G-CSF fusion protein with an appropriate peptide linker between the hG-CSF and Fc moieties (hG-CSF-L-Fc) is more active than rhG-CSF, with *in vitro* activity at least 2-fold as that of rhG-CSF on a molar basis. It is discovered according to this invention that an added peptide linker present between hG-CSF and a human IgG Fc variant enhances the *in vitro* biological activity of the hG-CSF-L-Fc molecule in two ways: (1) keeping the Fc region away from the G-CSF-Rc binding sites on G-CSF, and (2) keeping one G-CSF from the other G-CSF domain, so both G-CSF domains can interact with G-CSF-Rc on the granulocyte procursor cells independently. For the present invention, a flexible peptide linker of about 20 or fewer amino acids in length is preferred. More preferably, the peptide linker should have at least two amino acids in length. Furthermore, it is even

more preferable to use a peptide linker comprising two or more of the following amino acids: glycine, serine, alanine, and threonine.

[0007] The Fc region of human immunoglobulins plays a significant role in immune defense for the elimination of pathogens. Effector functions of IgG are mediated by the Fc region through two major mechanisms: (1) binding to the cell surface Fc receptors ($Fc_\gamma Rs$) can lead to ingestion of pathogens by phagocytosis or lysis by killer cells *via* the antibody-dependent cellular cytotoxicity (ADCC) pathway, or (2) binding to the C1q part of the first complement component C1 initiates the complement-dependent cytotoxicity (CDC) pathway, resulting in the lysis of pathogens. Among the four human IgG isotypes, IgG1 and IgG3 are effective in binding to $Fc_\gamma R$. The binding affinity of IgG4 to $Fc_\gamma R$ is an order of magnitude lower than that of IgG1 or IgG3, while binding of IgG2 to $Fc_\gamma R$ is below detection. Human IgG1 and IgG3 are also effective in binding to C1q and activating the complement cascade. Human IgG2 fixes complement poorly, and IgG4 appears quite deficient in the ability to activate the complement cascade (see, for example, Jefferis et al., *Immunol. Rev.*, 163:59-76, 1998). For therapeutic use in humans, it is essential that when hG-CSF-L-Fc binds to G-CSF-Rc on the surface of the progenitor cells or other cells of the myeloid lineage, the Fc region of the fusion protein will not mediate undesirable effector functions, leading to the lysis or removal of these cells. Accordingly, the Fc region of hG-CSF-L-Fc must be of a non-lytic nature, *i. e.* the Fc region must be inert in terms of binding to $Fc_\gamma Rs$ and C1q for the triggering of effector functions. It is clear that none of the naturally occurring IgG isotypes is suitable for use to produce the hG-CSF-L-Fc fusion protein. To obtain a non-lytic Fc, certain amino acids of the natural Fc region have to be mutated for the attenuation of the effector functions.

[0008] By comparing amino acid sequences of human and murine IgG isotypes, a portion of Fc near the N-terminal end of the CH2 domain is implicated to play a role in the binding of IgG Fc to $Fc_\gamma Rs$. The importance of a motif at positions 234 to 237 has been demonstrated using genetically engineered antibodies (see, for example, Duncan et al., *Nature*, 332:563-564, 1988). The numbering of the amino acid residues is according to the EU index as described in Kabat et al. (in *Sequences of Proteins of*

Immunological Interest, 5th Edition, United States Department of Health and Human Services, 1991). Among the four human IgG isotypes, IgG1 and IgG3 bind $Fc_\gamma Rs$ the best and share the sequence Leu234-Leu-Gly-Gly237 (only IgG1 is shown in Figure 1). In IgG4, which binds $Fc_\gamma Rs$ with a lower affinity, this sequence contains a single amino acid substitution, Phe for Leu at position 234. In IgG2, which does not bind $Fc_\gamma Rs$, there are two substitutions and a deletion leading to Val234-Ala-Gly237 (Figure 1). To minimize the binding of Fc to $Fc_\gamma R$ and hence the ADCC activity, Leu235 in IgG4 has been replaced by Ala (see, for example, Hutchins et al., *Proc. Natl. Acad. Sci. USA*, 92:11980-11984, 1995). IgG1 has been altered in this motif by replacing Glu233-Leu-Leu235 with Pro233-Val-Ala235, which is the sequence from IgG2. This substitution resulted in an IgG1 variant devoid of $Fc_\gamma R$-mediated ability to deplete target cells in mice (see, for example, Isaacs et al., *J. Immunol.*, 161: 3862-3869, 1998).

[0009] A second portion that appears to be important for both $Fc_\gamma R$ and C1q binding is located near the carboxyl-terminal end of CH2 domain of human IgG (see, for example, Duncan et al., *Nature*, 332:738-740, 1988). Among the four human IgG isotypes, there is only one site within this portion that shows substitutions: Ser330 and Ser331 in IgG4 replacing Ala330 and Pro331 present in IgG1, IgG2, and IgG3 (Figure 1). The presence of Ser330 does not affect the binding to $Fc_\gamma R$ or C1q. The replacement of Pro331 in IgG1 by Ser virtually abolished IgG1 ability to C1q binding, while the replacement of Ser331 by Pro partially restored the complement fixation activity of IgG4 (see, for example, Tao et al., *J. Exp. Med.*, 178:661-667, 1993; Xu et al., *J. Biol. Chem.*, 269:3469-3474, 1994).

[0010] We discover that at least three Fc variants (vFc) can be designed and/or used for the production of hG-CSF-L-vFc fusion proteins (Figure 1). Human IgG2 Fc does not bind $Fc_\gamma R$ but showed weak complement activity. An $Fc_{\gamma 2}$ variant with Pro331Ser mutation should have less complement activity than natural $Fc_{\gamma 2}$ while remain as a non-binder to $Fc_\gamma R$. IgG4 Fc is deficient in activating the complement cascade, and its binding affinity to $Fc_\gamma R$ is about an order of magnitude lower than that of the most active isotype, IgG1. An $Fc_{\gamma 4}$ variant with Leu235Ala mutation should exhibit minimal

effector functions as compared to the natural $Fc_{\gamma 4}$. The $Fc_{\gamma 1}$ variant with Leu234Val, Leu235Ala and Pro331Ser mutations also will exhibit much less effector functions than the natural $Fc_{\gamma 1}$. These Fc variants are more suitable for the preparation of the G-CSF fusion proteins than naturally occurring human IgG Fc. It is possible that other replacements can be introduced for the preparation of a non-lytic Fc without compromising the circulating half-life or causing any undesirable conformational changes.

[0011] There are many advantages with the present invention. The increased activity and prolonged presence of the hG-CSF-L-vFc fusion protein in the serum can lead to lower dosages as well as less frequent injections. Less fluctuations of the drug in serum concentrations also means improved safety and tolerability. Less frequent injections may result in better patient compliance and quality of life. The hG-CSF-L-vFc fusion protein containing a non-lytic Fc variant will therefore contribute significantly to the management of a variety of conditions associated with an impaired immune or hematopoietic system, including cancer chemotherapy, leukemias, anemias AIDS, bone marrow transplantation, and chronic neutropenias.

Summary of the invention

[0012] One aspect of the present invention relates to an hG-CSF-L-vFc fusion protein. This hG-CSF-L-vFc fusion protein comprises hG-CSF, a peptide linker (denoted by L), and a human IgG Fc variant (denoted by vFc). It is preferable to use a flexible peptide linker of about 20 or fewer, more preferably to about 2, amino acids in length and the flexible peptide linker contains or comprises of two or more of amino acids selected from the group consisting of glycine, serine, alanine, and threonine. The IgG Fc variant is of non-lytic nature and contains amino acid mutations as compared to naturally occurring IgG Fc.

[0013] It is another embodiment of the present invention that the human Ig Fc comprises a hinge, CH2, and CH3 domains of human IgG, such as human IgG1, IgG2, and IgG4. The CH2 domain contains amino acid mutations at positions 228, 234,

235, and 331 (defined by the EU numbering system). It is believed that these amino acid mutations serve to attenuate the effector functions of Fc.

[0014] In yet another embodiment of the present invention, a method is disclosed for making or producing such recombinant fusion proteins from a mammalian cell line such as a CHO-derived cell line. Growing transfected cell lines under conditions such that the recombinant fusion protein is expressed in its growth medium in excess of 10, preferably 30, μg per million cells in a 24 hour period. These hG-CSF-L-vFc fusion proteins are characterized by and exhibit increased/enhanced biological activity, preferably at least two fold (2X) *in vitro* activity, on a molar basis, relative to that of rhG-CSF and extended serum half-life without undesirable side effects, leading to improved pharmacokinetics and pharmacodynamics, thus lower dosages and fewer injections would be needed to achieve similar efficacies.

[0015] A further embodiment of the present invention provides a method for making a recombinant fusion protein comprising hG-CSF, a flexible peptide linker, and a human IgG Fc variant, which method comprises: (a) generating a CHO-derived cell line; (b) growing the cell line under conditions the recombinant fusion protein is expressed in its growth medium in excess of 10 μg, preferably 30μg, per million (10^6) cells in a 24 hour period; and (c) purifying the expressed protein from step (b), wherein the recombinant fusion protein is characterized by and exhibits an enhanced *in vitro* biological activity of at least 2 fold (2X) relative to that of rhG-CSF on a molar basis. In this case, preferably, the flexible peptide linker containing or comprising about 20 or fewer, but not fewer than 2, amino acids is present between hG-CSF and the human IgG Fc variant; and the flexible peptide linker comprises two or more amino acids selected from the group consisting of glycine, serine, alanine, and threonine; and wherein the human IgG Fc variant comprises a hinge, CH2, and CH3 domains selected from the group consisting of human IgG2 with Pro331Ser mutation, human IgG4 with Ser228Pro and Leu235Ala mutations, and human IgG1 with Leu234Val, Leu235Ala, and Pro331Ser mutations.

Brief descriptions of the drawings

[0016] Figure 1 shows the amino acid sequence alignment from the hinge and CH2 regions of human IgG1, IgG2, IgG4 and their variants. Three portions are compared: amino acid position 228, 234-237, and 330-331. Amino acid mutations of the variants are indicated in bold italics. The EU numbering system is used for the amino acid residues.

[0017] Figure 2 shows the nucleotide sequence and deduced amino acid sequence of (A) hG-CSF-L-v$Fc_{\gamma 2}$, (B) hG-CSF-L-v$Fc_{\gamma 4}$, and (C) hG-CSF-L-v$Fc_{\gamma 1}$ as the *Hin*dIII-*Eco*RI fragment in the respective phGFP expression vector. Amino acid residues -30 to -1 is the leader peptide of human G-CSF. The mature protein contains human G-CSF (amino acid residues 1 to 174), a peptide linker (amino acid residues 175 to 190), and a Fc variant (amino acid residues 191 to 418 of v$Fc_{\gamma 2}$, 191 to 419 of v$Fc_{\gamma 4}$, and 191 to 417 of v$Fc_{\gamma 1}$). In the Fc regions, nucleotide and corresponding amino acid mutations in bold are also underlined.

Detailed description of the invention

1. Construction of the gene encoding the hG-CSF-L-v$Fc_{\gamma 2}$ fusion protein

[0018] A fusion protein is assembled from several DNA segments. The gene encoding the leader peptide and mature protein of human G-CSF is obtained by reverse transcription and polymerase chain reaction (PCR) using RNA prepared from the human bladder carcinoma 5637 cell line. For the convenience of cloning, SEQ ID NO:1 (Table 1), which incorporates a restriction enzyme cleavage site (*Hin*dIII) is used as the 5' oligonucleotide primer. Table 1 shows the sequences of oligonucleotides used for the cloning of the hG-CSF-L-vFc fusion proteins. The 3' primer (SEQ ID NO:2) eliminates the G-CSF termination codon and incorporates a *Bam*HI site. The resulting DNA fragments of approximately 600 bp in length are inserted into a holding vector such as pUC19 at the *Hin*dIII and *Bam*HI sites to give the phGCSF plasmid. The sequence of the human G-CSF gene is confirmed by DNA sequencing.

[0019] The gene encoding the Fc region of human IgG2 ($Fc_{\gamma 2}$) is obtained by reverse transcription and PCR using RNA prepared from human leukocytes and appropriate 5' (SEQ ID NO:3) and 3' (SEQ ID NO:4) primers. Resulting DNA fragments of $Fc_{\gamma 2}$ containing complete sequences of the hinge, CH2, and CH3 domains of IgG2 will be used as the template to generate the $Fc_{\gamma 2}$ Pro331Ser variant ($vFc_{\gamma 2}$) in which Pro at position 331 of $Fc_{\gamma 2}$ is replaced with Ser. To incorporate this mutation, two segments are produced and then assembled by using the natural $Fc_{\gamma 2}$ as the template in overlapping PCR. The 5' segment is generated by using SEQ ID NO:3 as the 5' primer and SEQ ID NO:5 as the 3' primer. The 3' segment is generated by using SEQ ID NO:6 as the 5' primer and SEQ ID NO:4 as the 3' primer. These two segments are then joined at the region covering the Pro331Ser mutation by using SEQ ID NO:7 as the 5' primer and SEQ ID NO:4 as the 3' primer. The SEQ ID NO:7 primer contains sequences encoding a 16-amino acid Gly-Ser peptide linker including a *Bam*HI restriction enzyme site. The resulting DNA fragments of approximately 700 bp in length are inserted into a holding vector such as pUC19 at the *Bam*HI and *Eco*RI sites to give the pL-vFcγ2 plasmid. The sequence of the gene is confirmed by DNA sequencing.

[0020] To prepare the hG-CSF-L-$vFc_{\gamma 2}$ fusion gene, the hG-CSF fragment is excised from the phGCSF plasmid with *Hin*dIII and *Bam*HI and is purified by agarose gel electrophoresis. The purified fragment is then inserted to the 5'-end of the peptide linker in the pL-vFcγ2 plasmid to give the phG-CSF-L-vFcγ2 plasmid. The fusion gene comprises hG-CSF, a Gly-Ser peptide linker and the $Fc_{\gamma 2}$ variant gene.

[0021] The presence of a peptide linker, preferably a flexible linker, between (and chemically bound to both) the hG-CSF and Fc moieties increases the flexibility of the hG-CSF domains and enhances its biological activity. For the present invention, a peptide linker of about 20 or fewer amino acids in length is preferred. While a single amino acid is within the scope of the present invention, it is preferred to have a flexible peptide linker of about 20 to about 2 amino acids in length. Peptide linker containing or comprising of two or more of amino acids selected from the group consisting of glycine, serine, alanine, and threonine can be used preferably. An example of the peptide linker contains Gly-Ser peptide building blocks, such as

GlyGlyGlyGlySer. Figure 2A shows a fusion gene containing sequences encoding hG-CSF, a 16-amino acid peptide linker (GlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer, SEQ ID NO: 23), and the $Fc_{\gamma 2}$ Pro331Ser variant, and its corresponding amino acid sequence (SEQ ID NO: 18).

[0022] The complete gene encoding the hG-CSF-L-vFc fusion protein is then inserted at the *Hin*dIII and *Eco*RI sites of a mammalian expression vector, such as pcDNA3 (Invitrogen). The final expression vector plasmid, named phGFP2, contains the cytomegalovirus early gene promoter-enhancer that is required for high level expression in mammalian cells. The plasmid also contains selectable markers to confer ampicillin resistance in bacteria, and G418 resistance in mammalian cells. In addition, the phGFP2 expression vector contains the dihydrofolate reductase (DHFR) gene to enable the co-amplification of the hG-CSF-L-vFcγ2 fusion gene and the DHFR gene in the presence of methotrexate (MTX) when the host cells are deficient in the DHFR gene expression (see, for example, US patent 4,399,216).

2. Construction of the gene encoding the hG-CSF-L-vFc$_{\gamma 4}$ fusion protein

[0023] Human IgG4 is observed partly as half antibody molecules due to the dissociation of the inter-heavy chain disulfide bonds in the hinge domain. This is not seen in the other three human IgG isotypes. A single amino acid substitution replacing Ser228 with Pro, which is the residue found at this position in IgG1 and IgG2, leads to the formation of IgG4 complete antibody molecules (see, for example, Angal et al., *Molec. Immunol.*, 30:105-108, 1993; Owens et al., *Immunotechnology,* 3:107-116, 1997; US Patent 6,204,007). The $Fc_{\gamma 4}$ variant containing Leu235Ala mutation for the minimization of FcR binding will also give rise to a homogeneous fusion protein preparation with this additional Ser228Pro mutation.

[0024] The gene encoding the Fc region of human IgG4 ($Fc_{\gamma 4}$) is obtained by reverse transcription and PCR using RNA prepared from human leukocytes andappropriate 5' primer (SEQ ID NO:8) and 3' primer (SEQ ID NO:9). Resulting DNA fragments of $Fc_{\gamma 4}$ containing complete sequences of the hinge, CH2, and CH3

domains of IgG4 is used as the template to generate the $Fc_{\gamma 4}$ variant with Ser228Pro and Leu235Ala mutations ($vFc_{\gamma 4}$) in which Ser228 and Leu235 have been replaced with Pro and Ala, respectively. The CH2 and CH3 domains are amplified using the 3' primer (SEQ ID NO:9) and a 5' primer containing the Leu235Ala mutation (SEQ ID NO:10). This amplified fragment, together with a synthetic oligonucleotide of 60 bases in length (SED ID NO:11) containing both Ser228Pro and Leu235Ala mutations, are joined in PCR by using SEQ ID NO:12 as the 5' primer and SEQ ID NO:9 as the 3' primer. The SEQ ID NO:12 primer contains sequences encoding a 16-amino acid Gly-Ser peptide linker including the *Bam*HI site. The resulting DNA fragments of approximately 700 bp in length are inserted into a holding vector such as pUC19 at the *Bam*HI and *Eco*RI sites to give the pL-vFcγ4 plasmid. The sequence of the gene is confirmed by DNA sequencing.

[0025] To prepare the hG-CSF-L-$vFc_{\gamma 4}$ fusion gene, the hG-CSF fragment is excised from the phGCSF plasmid with *Hin*dIII and *Bam*HI and then inserted to the 5'-end of the peptide linker in the pL-vFcγ4 plasmid to give the phG-CSF-L-vFcγ4 plasmid. This fusion gene comprising hG-CSF, a 16-amino acid Gly-Ser peptide linker and the $Fc_{\gamma 4}$ variant gene is then inserted at the *Hin*dIII and *Eco*RI sites of a mammalian expression vector, such as pcDNA3 (Invitrogen), as described for the hG-CSF-L-$vFc_{\gamma 2}$ fusion protein. The final expression vector plasmid is designated as phGFP4. Figure 2B shows a fusion gene containing sequences encoding hG-CSF, a 16-amino acid peptide linker (GlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer), and the $Fc_{\gamma 4}$ variant with Ser228Pro and Leu235Ala mutations, and its corresponding amino acid sequence (SEQ ID NO: 20).

3. Construction of the gene encoding the hG-CSF-L-$vFc_{\gamma 1}$ fusion protein

[0026] The hinge domain of human IgG1 heavy chain contains 15 amino acid residues (GluProLysSerCysAspLysThrHisThrCysProProCysPro, SEQ ID NO: 24) including 3 cysteine residues. Out of these 3 cysteine residues, the 2nd and 3rd are involved in the formation of disulfide bonding between two heavy chains. The 1st cysteine residue is involved in the disulfide bonding to the light chain of IgG. Since

there is no light chain present in the Fc fusion protein molecule, this cysteine residue may pair with other cysteine residues, leading to nonspecific disulfide bonding. The hinge domain of $Fc_{\gamma 1}$ can be truncated to eliminate the 1st cysteine residue (AspLysThrHisThrCysProProCysPro). The gene encoding the $Fc_{\gamma 1}$ region is obtained by reverse transcription and PCR using RNA prepared from human leukocytes and appropriate 5' primer (SEQ ID NO:13) and 3' primer (SEQ ID NO:4). Resulting DNA fragments containing the truncated hinge and complete sequences of CH2 and CH3 domains of $Fc_{\gamma 1}$ is used as the template to generate the $Fc_{\gamma 1}$ variant with Leu234Val, Leu235Ala, and Pro331Ser mutations ($vFc_{\gamma 1}$), and its corresponding amino acid sequence (SEQ ID NO: 22).

[0027] One way to incorporate these mutations is as follows: two segments are produced and then assembled by using the natural $Fc_{\gamma 1}$ as the template in overlapping PCR. The 5' segment is generated by using SEQ ID NO:14 as the 5' primer and SEQ ID NO:5 as the 3' primer. This 5' primer contains the Leu234Val, Leu235Ala mutations and the 3' primer contains the Pro331Ser mutation. The 3' segment is generated by using SEQ ID NO:6 as the 5' primer and SEQ ID NO:4 as the 3' primer. These 5' and 3' segments are then joined at the region covering the Pro331Ser mutation by using SEQ ID NO:14 as the 5' primer and SEQ ID NO:4 as the 3' primer. This amplified fragment of approximately 650 bp in length, together with a synthetic oligonucleotide of 55 bases (SED ID NO:15) containing Leu234Val and Leu235Ala, are joined in PCR by using SEQ ID NO:16 as the 5' primer and SEQ ID NO:4 as the 3' primer. The SEQ ID NO:16 primer contains sequences encoding a 16-amino acid Gly-Ser peptide linker including the *Bam*HI site. The resulting DNA fragments of approximately 700 bp in length are inserted into a holding vector such as pUC19 at the *Bam*HI and *Eco*RI sites to give the pL-vFcγ1 plasmid. The sequence of the gene is confirmed by DNA sequencing.

[0028] To prepare the hG-CSF-L-$vFc_{\gamma 1}$ fusion gene, the hG-CSF fragment is excised from the phGCSF plasmid with *Hin*dIII and *Bam*HI and insertedto the 5'-end of the peptide linker in the pL-vFcγ1 plasmid to give the phG-CSF-L-vFcγ1 plasmid. The fusion gene comprising hG-CSF, a 16-amino acid Gly-Ser peptide linker,

and the $Fc_{\gamma 1}$ variant gene is then inserted at the *Hin*dIII and *Eco*RI sites of a mammalian expression vector, such as pcDNA3 (Invitrogen), as described for the hG-CSF-L-v$Fc_{\gamma 2}$ fusion protein. The final expression vector plasmid is designated as phGFP1. Figure 2C shows a fusion gene containing sequences encoding hG-CSF, a 16-amino acid peptide linker (GlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer), and the $Fc_{\gamma 1}$ variant with Leu234Val, Leu235Ala and Pro331Ser mutations), and its corresponding amino acid sequence (SEQ ID NO: 22).

4. Expression of the fusion protein in transfected cell lines

[0029] Two different rhG-CSF have been produced: a glycosylated form produced in Chinese Hamster Ovary (CHO) cells and a nonglycosylated form produced in bacteirial cells. Glycosylated rhG-CSF contains O-linked oligosaccharides attached to the threonine amino acid residue at position 133, accounting for approximately 4% of its molecular weight. The carbohydrate chain contributes to the stabilization of the protein molecule by suppressing polymerization and conformational changes (Oh-eda et al., *J. Biol. Chem.,* 265:11432-11435, 1990). In *in vitro* studies using rhG-CSF, the glycosylated form produced in CHO cells is biologically more active than the nonglycosylated form produced in bacteirial cells (Nissen, *Eur. J. Cancer*, 30A Suppl 3:S12-S14, 1994). Furthermore, rhG-CSF derived from CHO cells was shown to be indistinguishable from its natural counterpart in terms of structural characteristics and biological activity (Kubota et al., *Biochem.*(Tokyo), 107:486-492, 1990). In randomized crossover studies in healthy volunteers, glycosylated rhG-CSF has been found to be 25 to 30% more potent than the nonglycosylated rhG-CSF on a weight for weight basis in the mobilization of peripheral blood progenitor cells (see, for example, Hoglund, *Med. Oncol.*, 15:229-233, 1998; Hoglund et al., *Eur. J. Haematol.*, 59:177-183, 1997). To obtain the protein most suitable for clinical use, the hG-CSF-L-vFc fusion protein will be produced in CHO cells as follows.

[0030] The recombinant phGFP1, phGFP2 or phGFP4 expression vector plasmid is transfected into a mammalian host cell line to achieve the expression of the hG-CSF-L-vFc fusion protein. For stable high levels of expression, a preferred host cell

line is CHO cells deficient in the DHFR enzyme (see, for example, US patent 4,818,679). A preferred method of transfection is electroporation. Other methods, including calcium phosphate co-precipitation, lipofectin, and protoplast fusion, can also be used. For electroporation, 10 μg of plasmid DNA linearized with *Bsp*CI is added to 2 to 5 x 10^7 cells in a cuvette using Gene Pulser Electroporator (Bio-Rad Laboratories, Hercules, CA) set at an electric field of 250 V and a capacitance of 960 μFd. Two days following the transfection, the media are replaced with growth media containing 0.8 mg/ml of G418. Transfectants resistant to the selection drug are tested for the secretion of the fusion protein by anti-human IgG Fc ELISA. Quantitation of the expressed fusion protein can also be carried out by ELISA using anti-hG-CSF assays. The wells producing high levels of the Fc fusion protein are subcloned by limiting dilutions on 96-well tissue culture plates.

[0031] To achieve higher levels of the fusion protein expression, co-amplification is preferred by utilizing the gene of DHFR that can be inhibited by the MTX drug. In growth media containing increasing concentrations of MTX, the transfected fusion protein gene is co-amplified with the DHFR gene. Transfectants capable of growing in media with up to 1 μg/ml of MTX are again subcloned by limiting dilutions. The subcloned cell lines are further analyzed by measuring the secretion rates. Several cell lines yielding secretion rate levels over about 10, preferably about 30 μg/10^6 [*i.e.* million]cells/24h, are adapted to suspension culture using serum-free growth media. The conditioned media are then used for the purification of the fusion protein.

5. Purification and characterization of the fusion protein

[0032] Conditioned media containing the fusion protein are titrated with 1 N NaOH to a pH of 7 to 8 and filtered through a 0.45 micron cellulose nitrate filter. The filtrate is loaded onto a Prosep A column equilibrated in phospate-buffered saline (PBS). After binding of the fusion protein to Prosep A, the flow-through fractions are discarded. The column is washed with PBS until OD at 280 nm is below 0.01. The bound fusion protein is then eluted with 0.1 M citrate buffer at pH 3.75. After neutralizing with 0.4 volume of 1 M K_2HPO_4, fractions containing purified protein are pooled and dialyzed

against PBS. The solution is then filtered through a 0.22 micron cellulose nitrate filter and stored at 4°C. The molecular weight of purified hG-CSF-L-vFc protein is in the range of 90 and 110 kDa by SDS-PAGE under non-reducing conditions. Under reducing conditions, the purified protein migrates around approximately 50 kDa. The fusion protein is quantitated by BCA protein assay using BSA as the standard.

6. *In vitro* biological assays

[0033] Supernatants of transfectants or purified proteins can be tested for their ability to stimulate the proliferation of murine myeloblastic NFS-60 cells (Shirafuji et al., *Exp. Hematol.*, 17:116-119, 1989). NFS-60 cells are responsive to rhG-CSF but not to rhGM-CSF or hM-CSF. The cells are maintained in growth medium (RPMI-1640 medium containing 10% FCS and murine IL-3 at 1 ng/ml). Log phase NFS-60 cells are collected and washed with assay medium (growth medium without murine IL-3). A total of 1 x 10^4 cells per sample of NFS-60 in 50 µl is added to each well of a 96-well tissue culture plate. The cells are incubated with 50 µl of assay media containing various concentrations of the hG-CSF-L-vFc fusion protein or the rhG-CSF control from 0.01 to 100 nM each. The plate is kept at 37°C and 5% CO_2 in a humidified incubator for 4 days before 10 µl of MTT (2.5 mg/ml in PBS) is added to each well. After 4 h, the cells and formazan are solubilized by adding 100µl per well of 10% SDS in 0.01 N HCl. The plate is then read at 550 nm with the reference beam set at 690 nm. The OD reading is plotted against the concentration of rhG-CSF or the fusion protein. The inflection point of the sigmoidal curve represents the concentration at which 50% of the maximal effect, ED50, is induced. The biological activity of hG-CSF-L-vFc relative to that of rhG-CSF can therefore be compared quantitatively. Preferably, the recombinant fusion proteins should be characterized by and exhibit an enhanced activity of at least 2 fold (2X) relative to that of rhG-CSF on a molar basis. In one embodiment of the present invention, the specific activity of the hG-CSF-L-vFc fusion protein is in the range of about 1.5 to about 6.0 x 10^9 units/µmole, compared to about 0.75 to about 3.0 x 10^9 units/µmole for rhG-CSF based on this cell proliferation assay.

[0034] Supernatants of transfectants or purified proteins can also be tested for their ability to stimulate the proliferation and differentiation of human bone marrow progenitor cells to form colonies, granulocyte-macrophage colony forming unit (CFU-GM). The procedure is as follows. Light-density cells from human bone marrow centrifuged over Ficoll-Pague are washed and resuspended at 1 x 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) containing 5% FCS. These cells containing enriched progenitor cells are incubated in a tissue culture dish overnight at 37°C and 5% CO_2 to remove all adherent cells including monocytes, macrophages, endothelial cell, and fibroblasts. Cells in suspension are then adjusted to 1 x 10^5 cells/ml in IMDM containing 5% FCS. For the assay, 0.3 ml of cells, 15 µl of stem cell factor at 20 µg/ml, 2.4 ml of methylcellulose, and 0.3 ml of media containing several concentrations of hG-CSF-L-vFc (or rhG-CSF control) are mixed. One ml of this cell mixture is plated on a 35-mm petri dish. The dishes are then kept at 37°C and 5% CO_2 for 10 to 14 d before the colonies are counted. A dose responsive curve can be plotted against the concentrations of hG-CSF-L-vFc relative to those of rhG-CSF.

7. *In vivo* pharmacokinetic studies in rats

[0035] Fisher rats (Harlan Bioproducts for Science, Indianapolis, IN) with an average body weight of about 500 g are injected i.v. through the tail vein or s.c. with 100 units of rhG-CSF or the hG-CSF-L-vFc fusion protein. An equal volume of PBS is injected as a control. Serial 0.5-ml samples are taken through retro-orbital bleeds at different points (0, 0.2, 1, 4, 24, 48, 96, and 168 h) after injection. There are 3 rats for each time point. Whole blood is collected into tubes containing anticoagulant, cells are removed, and plasma is frozen at -70°C until assay is carried out.

[0036] Serum samples are used for NFS-60 cell assays, which measure the activity of hG-CSF-mediated cell proliferation. A total of 1 x 10^4 cells per sample of NFS-60 in 50 µl is added to each well of a 96-well tissue culture plate. The cells are incubated with 50 µl of assay media containing various concentrations of titrated blood samples. The plate is kept at 37°C and 5% CO_2 in a humidified incubator for 4 days. Viable cells are stained with 10 µl of MTT (2.5 mg/ml in PBS). After 4 h, the cells and

formazan are solubilized by adding 100 µl per well of 10% SDS in 0.01 N HCl. The plate is then read at 550 nm with the reference beam set at 690 nm. The activities of serum samples are plotted against time points for the calculation of the circulation time. The activity of hG-CSF-L-vFc decreases much slower than that of the rhG-CSF control, indicating the longer circulating half-life of the fusion protein in rats.

[0037] The examples described above are for illustration purposes only. They are not intended and should not be interpreted to limit either the scope or the spirit of this invention. It can be appreciated by those skilled in the art that many other variations or substitutes can be used as equivalents for the purposes of this invention, which is defined solely by the written description and the following claims.

Table 1. Sequences of Oligonucleotides.

SEQ ID NO:1
5′-cccaagcttcccagacccatggctggacct-3′

SEQ ID NO:2
5′-cggatccgggctgggcaaggtggcgta-3′

SEQ ID NO:3
5′-gagcgcaaatgttgtgtcga-3′

SEQ ID NO:4
5′-ggaattctcatttacccggagacaggga-3′

SEQ ID NO:5
5′-tggttttctcgatggaggctgggaggcct-3′

SEQ ID NO:6
5′-aggcctcccagcctccatcgagaaaacca-3′

SEQ ID NO:7
5′-cggatccggtggcggttccggtggaggcggaagcggcggtggaggatcag
agcgcaaatgttgtgtcga-3′

SEQ ID NO:8
5′-gagtccaaatatggtccccca-3′

SEQ ID NO:9
5′-ggaattctcatttacccagagacaggga-3′

SEQ ID NO:10
5′-cctgagttcgcgggggggacca-3′

SEQ ID NO:11
5′-gagtccaaatatggtcccccatgcccaccatgcccagcacctgagttcgcgg
ggggacca-3′

SEQ ID NO:12
5′-cggatccggtggcggttccggtggaggcggaagcggcggtggaggatcagag
tccaaatatggtccccca-3′

SEQ ID NO:13
5′-gacaaaactcacacatgccca-3′

SEQ ID NO:14
5′-acctgaagtcgcgggggggaccgt-3′

SEQ ID NO:15
5'-gacaaaactcacacatgcccaccgtgcccagcacctgaagtcgcgggggac
cgt -3'

SEQ ID NO:16
5'-cggatccggtggcggttccggtggaggcggaagcggcggtggaggatcagac
aaaactcacacatgccca-3'